

## EBNA3A Association with RBP-J $\kappa$ Down-Regulates *c-myc* and Epstein-Barr Virus-Transformed Lymphoblast Growth

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Epstein-Barr virus nuclear antigen protein 3A (EBNA3A) is one of four EBNAs (EBNA-2, EBNA1P, EBNA3A, and EBNA3C) through the cellular DNA sequence-specific transcription factor RBP-J $\kappa$ /CBF-1/CSL and are essential for conversion of primary B lymphocytes to lymphoblastoid cell lines (LCLs). In the present study, we investigated the effects of EBNA3A on EBNA2 activation of transcription in the IB4 LCL by conditionally overexpressing EBNA3A three- to fivefold. EBNA3A overexpression increased EBNA3A association with RBP-J $\kappa$ , did not change EBNA3C association with RBP-J $\kappa$  or EBNA or LMP1 expression, decreased EBNA2 association with RBP-J $\kappa$ , decreased *c-myc* expression, and caused G<sub>0</sub>/G<sub>1</sub> growth arrest with prolonged viability. Expression of the fusion protein MycERTM in cells with conditional EBNA3A overexpression restored cell cycle progression and caused apoptosis. In contrast, MycER in the same cells without EBNA3A overexpression enhanced cell proliferation and did not increase apoptosis. These data indicate that EBNA3A overexpression inhibits protection from *c-myc*-induced apoptosis. In assays of EBNA2- and RBP-J $\kappa$ -dependent transcription, EBNA3A amino acids 1 to 386 were sufficient for repression equivalent to that by wild-type EBNA3A, amino acids 1 to 124 were unimportant, amino acids 1 to 277 were insufficient, and a triple alanine substitution within the EBNA3A core RBP-J $\kappa$  binding domain was a null mutation. In reverse genetic experiments with IB4 LCLs, the effects of conditional EBNA3A overexpression on *c-myc* expression and proliferation did not require amino acids 524 to 944 but did require amino acids 278 to 524 as well as wild-type sequence in the core RBP-J $\kappa$  binding domain. The dependence of EBNA3A effects on the core RBP-J $\kappa$  interaction domain and on the more C-terminal amino acids (amino acids 278 to 524) required for efficient RBP-J $\kappa$  association strongly implicates RBP-J $\kappa$  in *c-myc* promoter regulation.

Epstein-Barr virus (EBV) is a gammaherpesvirus that causes lymphoproliferative diseases in immune-compromised people and rarely in otherwise healthy people (43). EBV infection of primary human B lymphocytes results in EBV expression of nuclear proteins EBNA1, -2, -LP, -3A, -3B, and -3C, integral membrane proteins LMP1, -2A, and -2B, small RNAs (EBERs), *Bam*HI A rightward transcripts that may encode three proteins, and perpetual lymphoblastoid cell growth (lymphoblastoid cell lines [LCLs]) (43, 64). Recombinant reverse genetic analyses indicate that EBNA1, -2, -3A, -3C, and -LP and LMP1 are necessary for LCL outgrowth and that most of the rest of the viral genome is not required.

EBNA2 (E2) and EBNA1P are the first viral gene products expressed in latent infection, and they coactivate transcription from a subset of cell and viral promoters (1, 28). E2 self-associates, associates with the sequence-specific DNA binding protein RBP-J $\kappa$ /CBF-1/CSL, and activates transcription from promoters containing RBP-J $\kappa$  (RBP) binding sites (27, 29, 32, 38, 39, 50, 71, 77). The E2 acidic activation domain interacts with transcription factors including TFIIB, TFIIF, p100, and p300/CBP, as well as with EBNA1P (28, 68–70, 75). E2 activates the cellular CD21, CD23, *c-fgr*, and *c-myc* promoters and

the viral EBNA and latent membrane protein (LMP) promoters (1, 12, 16, 21, 38, 40, 44, 73, 74). The EBNA and LMP promoters and the cellular CD21 and CD23 promoters have nearby RBP sites that are likely to be important for E2 responsiveness (16, 38, 52, 80).

Studies with a conditionally active E2 estrogen receptor fusion protein in an LCL indicate that *c-myc* is at least in part directly regulated by E2 (40). A constitutively active form of Notch, which also activates transcription through RBP, partially restored *c-myc* expression and growth in an LCL clone that was arrested following E2 inactivation, consistent with the hypothesis that RBP is a mediator of E2 activation of *c-myc* (25). Although overexpressed Notch could partially substitute for E2 in this cell line, and constitutively active Notch can be leukemogenic, other genetic data indicate that *c-myc* is complementary rather than downstream of Notch in leukemogenesis (24, 34).

Up-regulation of the EBNA promoter by E2 and EBNA1P results in their enhanced transcription and in transcription of EBNA3A, EBNA3B, EBNA3C, and EBNA1 (43). EBNA1 can further up-regulate the EBNA promoters (59), whereas EBNA3A, EBNA3B, and EBNA3C associate with RBP and could thereby limit the coactivating effects of E2 and EBNA1P (61, 82). Since other cellular factors, including PU.1/Spi-1 and AUF1, contribute to responsiveness at some promoters, the EBNA3 proteins would specifically inhibit E2 and EBNA1P activity, which is critically dependent on RBP (22, 38).

To investigate the potential role of the EBNA3 proteins in

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limiting E2- and EBNA1-mediated transcriptional activation, we have investigated the effects of increased EBNA3A (E3A) expression on viral and cellular gene expression in an LCL. In transient transfection assays, E3A was known to reduce RBP-dependent E2 activation of promoters (5, 10, 13, 45, 47, 61, 72, 82). Further, E3A, or even E3A with a deletion of the RBP interaction domain, fused to the GAL4 DNA binding domain can repress a promoter with upstream GAL4 DNA binding sites (5, 10, 72). However, E3A can also have transcriptional activating effects (10, 13, 49, 72). The IB4 LCL was used in these experiments because the E2 response element in the EBNA promoter is deleted and EBV gene expression is somewhat stabilized by integration of the EBV genome into cell DNA (31, 37, 76). Thus, increased EBNA3A expression in IB4 may have less effect on the expression of other EBV genes.

## MATERIALS AND METHODS

**Plasmids.** pSG5-FE3A was created by ligating a *PstI/EcoRV* fragment from pBS-EBNA3A into *PstI/EcoRV*-digested pSG5-FLAG (30), followed by replacement of the *PstI/SacI* fragment with a *PstI/SacI*-digested PCR product amplified with forward and reverse oligonucleotides 5'-AGTACTGCTGCAGACAAAATGGACAAGGAC-3' and 5'-TGTTACCCACGGAGCTCTGAACATACAAC C-3' to insert a *PstI* site 5' of the E3A open reading frame. The resulting construct expresses full-length E3A with an N-terminal FLAG epitope tag and was verified by sequencing. pJEF4-FE3A was constructed by ligating *EcoRI/BamHI* and *BamHI/EcoRI* fragments from pSG5-FE3A into *EcoRI/BamHI*-digested pJEF4 (20). An *EcoRI* fragment from pSG5-FE3A containing E3A codons 1 to 277 was cloned into the *EcoRI* sites of pJEF4 or pSG5-FLAG to generate pJEF4-F277 or pSG5-F277, respectively. pSG5-F302 was created by linker insertion of a stop codon into *SpeI/AgeI*-digested pSG5-FE3A by use of oligonucleotides 5'-CTAGCTAGTCTAGACAC-3' and 5'-CCGGGTGTCTAGACTAG-3'. pSG5-F386 was created by *AatII* collapse of pSG5-FE3A. pJEF4-F523 and pSG5-FLAG523 were constructed by ligating the *PstI/StuI* fragment from pSG5-FE3A into *PstI/HpaI*-digested pJEF4-FE3A or pSG5-FE3A, respectively. pSG5-F826 was made by insertion of a *PstI/HpaI* fragment of E3A into *PstI/EcoRV*-digested pSG5. pJEF4-Fdel was created by *BglII* collapse of pJEF4-FE3A, deleting codons 101 to 365; the deletion was subcloned into pSG5-FE3A to make pSG5-Fdel. pSG5-Fd124, expressing FLAG-tagged E3A amino acids 125 to 944, was made by excising a *BamHI/EcoRV* fragment from pSG5-FE3A, blunting the ends with T4 DNA polymerase, and ligating into pSG5-FLAG digested with *XhoI* and blunted with T4 polymerase. Alanine substitution mutation of amino acids T199, L200, and C202 in pJEF4-FE3A or pSG5-FE3A was carried out using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and complementary oligonucleotides 5'-CGTACATCTCCAGCGCCGACGTGGGCCACAGGTGGCCGTAGG-3' and 5'-CCTACGCCACCTGTGGCCC CAGTCCGCCCTGGAGATGTACG-3' according to the manufacturer's instructions to create pJEF4-FAAA or pSG5-FAAA, respectively. The resulting constructs were verified by sequencing. The pJEF3 plasmid encoding the tetracycline (TET) transactivator has been described previously (20). pSG5-mycER was constructed by inserting an *EcoRI* fragment from pBABE-mycER (51) into *EcoRI*-digested pSG5 (Stratagene). The pSG5-EBNA 2, pGK- $\beta$ gal, and pLucCp plasmids used for reporter assays have been described previously (22, 28, 30).

**Antibodies.** Anti-FLAG monoclonal antibodies M2 and M5 were purchased from Kodak. A fragment of E3A (amino acids 125 to 279) was fused to glutathione S-transferase (GST), expressed in *Escherichia coli*, purified by polyacrylamide gel electrophoresis (PAGE), and inoculated into rabbits to generate an antiserum to E3A (Pocono Rabbit Farms and Laboratory, Inc.), which was affinity purified as described by Youssoufian (78). The anti-EBNA1 monoclonal antibody was purchased from ABI. Monoclonal antibodies to E2 (PE2), EBNA1 (JF186), EBNA3C (A10), and LMP1 (S12) have been described previously (19, 53, 56). The polyclonal antiserum to RBP-Jk (St184) has been described by Johannsen et al. (39). Polyclonal antisera to c-Myc (N-262), cyclin D2 (C-17), p21 (C-19), and p53 (FL-393) and monoclonal anti-Myc (9E10) were purchased from Santa Cruz, and  $\alpha$ -actin antiserum (C4) was from ICN.

**Cell lines.** IB4, an EBV-transformed normal human cord blood LCL, with stable expression of the TET transactivator, has been described previously (7) and was electroporated with the pJEF4-derived plasmids described above, plated at 10,000 to 20,000 cells/well in 96-well plates, and selected in 0.8 mg of G418 (Gemini)/ml and 1  $\mu$ g of TET (Sigma)/ml. Several clones exhibited expression of

each FLAG fusion protein upon removal of TET from the culture medium, and two clones of each were selected for further study. BL-41, an EBV-negative Burkitt lymphoma cell line with stable expression of the TET transactivator, was generated as described by Cahir-McFarland et al. (7), and clones with stable conditional FE3A expression were derived as described above. To induce expression of wild-type and mutant FE3A proteins in IB4 clones, the culture medium containing TET was removed and cells were maintained either in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Gemini) (R10) (for induction) or in R10 plus 0.5  $\mu$ g of TET/ml for 24 to 48 h. Cells were washed in R10 and cultured at  $5 \times 10^4$  to  $10 \times 10^4$ /ml (day 0) in either R10 alone or R10 plus TET. One clone was selected for further study. pSG5-mycERTM was electroporated along with pBABE-Puro (kindly provided by T. Littlewood and G. Evans) into IB4 clone I2, plated at 25,000 to 50,000 cells/well in 96-well plates, and selected in 1 mg of puromycin (Sigma)/ml and 0.5  $\mu$ g of TET/ml. Clones with expression of the mycERTM fusion protein and conditional FE3A expression were identified, and one clone was selected for further study. FE3A was induced as described above, and following 2 days of induction, cultures were supplemented with 100 nM 4-hydroxytamoxifen (4-HT) to stabilize and activate MycERTM. BJAB is an EBV-negative Burkitt lymphoma cell line. All cells were maintained in R10 with 0.5  $\mu$ g of TET/ml unless otherwise specified.

**Growth and cell cycle analysis.** Cell numbers were determined with a hemacytometer, and approximately  $10^6$  cells were harvested, stained with propidium iodide (Molecular Probes), and analyzed on a FACS calibur (Becton Dickinson) by using CELLQUEST software as previously described (7).

**Immunoblotting.** Whole-cell lysates or other samples were prepared by solubilizing in sodium dodecyl sulfate (SDS) sample buffer and heating at 95°C for 10 min. Lysates corresponding to  $2.5 \times 10^5$  to  $5 \times 10^5$  cells per sample (unless otherwise indicated) were separated by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and probed overnight at 4°C with the indicated primary antibodies. Blots were subsequently incubated for 1 h with species-specific secondary antibodies conjugated to horseradish peroxidase (Santa Cruz) and diluted 1:5,000 in phosphate-buffered saline (PBS) supplemented with 1% milk. Membranes incubated with chemiluminescence reagent (NEN) were exposed to X-ray film (Kodak) to detect immunoreactive proteins.

**Immunoprecipitations.** Approximately  $3 \times 10^6$  cells from IB4 clones in which FE3A or FE3A mutant protein expression was induced or repressed were lysed in immunoprecipitation (IP) buffer (150 mM NaCl, 1% NP-40, 50 mM Tris [pH 7.4], 2 mM EDTA) supplemented with protease inhibitors (10  $\mu$ g of aprotinin/ml, 0.5 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M pepstatin, 1  $\mu$ M leupeptin) by vortexing, maintained on ice for 1 h, and centrifuged to remove insoluble debris. A portion of the supernatant was reserved for analysis, and the remainder was incubated with a 50% slurry of M2-conjugated Sepharose beads (Kodak), PE2 monoclonal antibody plus protein G-conjugated Sepharose (Pharmacia), or St184 antiserum plus protein A-conjugated Sepharose (Pharmacia) in IP buffer for 2 h at 4°C. Beads were washed four times with 1 ml of IP buffer at 4°C, and proteins were eluted with an equal volume of SDS sample buffer. A portion of each immunoprecipitate and the indicated percentage of the corresponding input lysate were analyzed by immunoblotting as described above.

**Surface expression of CD21 and CD23.** Approximately  $10^6$  cells were harvested, washed in PBS, and incubated with phycoerythrin-conjugated anti-CD21 or anti-CD23 antibodies (Pharmingen) diluted 10-fold in PBS for 30 min at 4°C. Cells were washed in PBS supplemented with 2% fetal bovine serum and analyzed on a FACScaliber by using CELLQUEST software.

**Northern blotting.** Total RNA was prepared from cells by using the TRIzol reagent (Invitrogen Life Technologies) according to the instructions of the manufacturer, and 20  $\mu$ g of each RNA sample was electrophoresed on formaldehyde gels and transferred to Zeta-probe membranes (Bio-Rad). Northern blots were hybridized overnight at 65°C with DNA probes labeled with  $^{32}$ P by using the Multi-Prime DNA Labeling kit (Amersham) and fragments of the human *c-myc* or *gapdh* cDNA overnight at 65°C. Radiolabeled RNA was visualized with a PhosphorImager using ImageQuant software (Molecular Dynamics).

**Confocal microscopy.** To analyze the nuclear localization of *c-myc*, live cells were streaked onto glass slides at 37°C. Cells were fixed in 1:1 methanol-acetone at -20°C for 10 min. Slides were air dried and incubated with 20% goat serum (Gemini) in a humidity chamber for 1 h at room temperature, followed by a 2-h incubation with monoclonal antibody 9E10 diluted 500-fold in PBS. Subsequently, cells were incubated with a 1:1,000 dilution of goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (Amersham) for 1 h at room temperature and were visualized with a PCM2000 (Nikon) coupled to a Zeiss microscope using SIMPLE32 software (C Imaging Systems).

**Reporter assays.** Reporter analysis was carried out as described previously (49). In brief, approximately  $10^7$  BJAB cells in log-phase growth were electro-

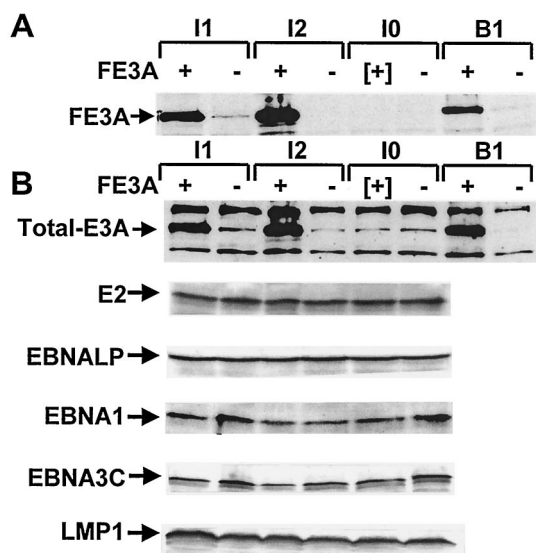


FIG. 1. Conditional three- to fivefold overexpression of FE3A in IB4 LCL clones I1 and I2 does not affect endogenous EBV latent gene expression. I1, I2, and BL41 clone B1 cells were cultured under conditions of FE3A induction (+) or repression (-) for 4 days. [+], culture of the control IB4 clone I0, which does not conditionally express FE3A, under induction conditions. (A) Conditional FE3A expression in I1, I2, and B1 was examined by Western blotting with FLAG epitope-specific monoclonal antibodies M2 and M5. (B) Western blot for EBV latent gene expression with a polyclonal E3A antiserum (total E3A), an EBV immune human serum (E2), or a monoclonal antibody to EBNA1, EBNA3C, or LMP1.

porated with 0.5  $\mu$ g of pGK- $\beta$ -gal and 1  $\mu$ g of pSG5-EBNA 2 alone or in combination with adjusted amounts of wild-type pSG5-FE3A or pSG5-FE3A mutant constructs. Cells were harvested for analysis 48 h posttransfection.

## RESULTS

**E3A overexpression in the IB4 LCL results in  $G_0/G_1$  arrest, without altering viral gene expression.** Initial attempts to isolate IB4 LCL clones with increased E3A expression were unsuccessful, consistent with the possibility that E3A overexpression has an adverse effect on viral or cellular gene expression. The putative effect of E3A overexpression appeared to be LCL specific, since multiple clones of BJAB, a non-EBV-infected human Burkitt tumor cell line, were derived that expressed E3A at levels 5- to 10-fold higher than LCLs (data not shown).

Clones of IB4 LCLs and of an EBV-negative Burkitt tumor cell line (BL41) were then derived in which expression of a FLAG epitope-tagged E3A (FE3A) was conditionally repressed by a TET-inhibited transactivator. A clone of BL41 (B1) and two clones of IB4 cells (I1 and I2) were selected which conditionally expressed FE3A at levels 3, 3, and 5 times higher, respectively, than endogenous IB4 E3A (Fig. 1 and data not shown). A clone of IB4 cells (I0) that did not express FE3A upon TET withdrawal was kept as a control. FE3A induction in I1 and I2 LCLs caused cell growth arrest, whereas B1 and I0 cell growth were unaffected by TET withdrawal (Fig. 2A). Further, I1 or I2 cells with FE3A expression repressed continued to grow, and their growth was indistinguishable from that of I0 cells. Induced FE3A expression in I1 and I2 cells

resulted in a  $\sim 25\%$  increase in  $G_0/G_1$  cells, a decrease in S and  $G_2/M$  cells from  $\sim 36\%$  to 15%, and a 2% increase in apoptotic cells relative to I1 and I2 cells with FE3A repressed (Fig. 2C and Table 1). TET withdrawal in I0 cells or FE3A induced expression in B1 cells had no effect on cell cycle distribution. Despite growth arrest, FE3A-induced I1 and I2 LCLs remained viable, excluded trypan blue, and remained diploid in DNA content for at least 14 days (data not shown).

The FE3A-induced growth arrest in I1 and I2 LCLs was somewhat dependent on cell density, fetal calf serum (FCS) concentration, and FE3A expression levels. At cell densities of  $>2 \times 10^5$ /ml the growth arrest was less evident, while decreasing the [FCS] to 1 or 0.1% enhanced FE3A-mediated growth arrest (data not shown). IB4 clones with lower levels of induced FE3A expression were intermediate in phenotype between I0 and I1 or I2 LCLs (data not shown). Thus, conditional three- to fivefold EBNA3A overexpression in IB4 LCLs causes a  $G_0/G_1$  growth arrest, which can be overridden by paracrine or exogenous growth factors, whereas similar levels of EBNA3A overexpression in BL41 cells, which have constitutive *c-myc* expression, have no effect on cell growth.

FE3A-induced overexpression in I1 and I2 LCLs did not affect EBNA1, E2, EBNA1P, or LMP1 levels over 4 days of FE3A induction, whereas cell growth ceased (Fig. 1B). In multiple experiments, EBNA3C expression was not consistently altered in I1, I2, or I0 cells grown under FE3A-inducing conditions (Fig. 1B and data not shown). The expression of these EBV latent infection proteins also did not change after longer periods of E3A overexpression and growth arrest (data not shown). Therefore, FE3A-mediated growth arrest was not secondary to down-regulation of essential EBV transforming proteins.

**Conditional FE3A overexpression down-regulates CD21, CD23, and *c-myc* expression.** FE3A induction in I1 or I2 LCL cells was accompanied by decreases of nearly 50% in cell surface CD21 and CD23 expression (Fig. 3). CD21 and CD23 are induced by E2 and have RBP binding sites that can affect transcriptional regulation (52, 80).

FE3A induction also resulted in reduced *c-Myc* protein and mRNA levels, whereas FE3A induction in B1 Burkitt lymphoma cells or TET withdrawal from I0 LCL cells did not affect *c-myc* expression (Fig. 4A and B). *c-Myc* up-regulates cyclin D2 and can down-regulate p21 (11). FE3A induction was associated with diminished cyclin D2 expression, somewhat increased p21 expression, and unaltered p53 expression, whereas TET withdrawal from I0 or B1 cell cultures had no effect on cyclin D2, p21, or p53 levels (Fig. 4B).

Consistent with decreased cyclin D2 expression and  $G_0/G_1$  arrest following FE3A overexpression in I1 and I2 cells, but not in I0 or B1 cells, increased hypophosphorylated pRb and p130 levels were evident in these cells (data not shown). Two immunoreactive forms of pRb and four immunoreactive forms of p130 were observed by immunoblotting in uninduced I1 and I2 cells, and in I0 and B1 cells under conditions of induction or repression. FE3A induction resulted in the loss of the more slowly migrating hyperphosphorylated form of pRb and the two slowest-migrating forms of p130. In addition, a single immunoreactive form of p107 protein was readily observed in I1, I2, I0, and B1 cells grown under conditions of repression but was barely detectable in I1 and I2 cells in which FE3A over-



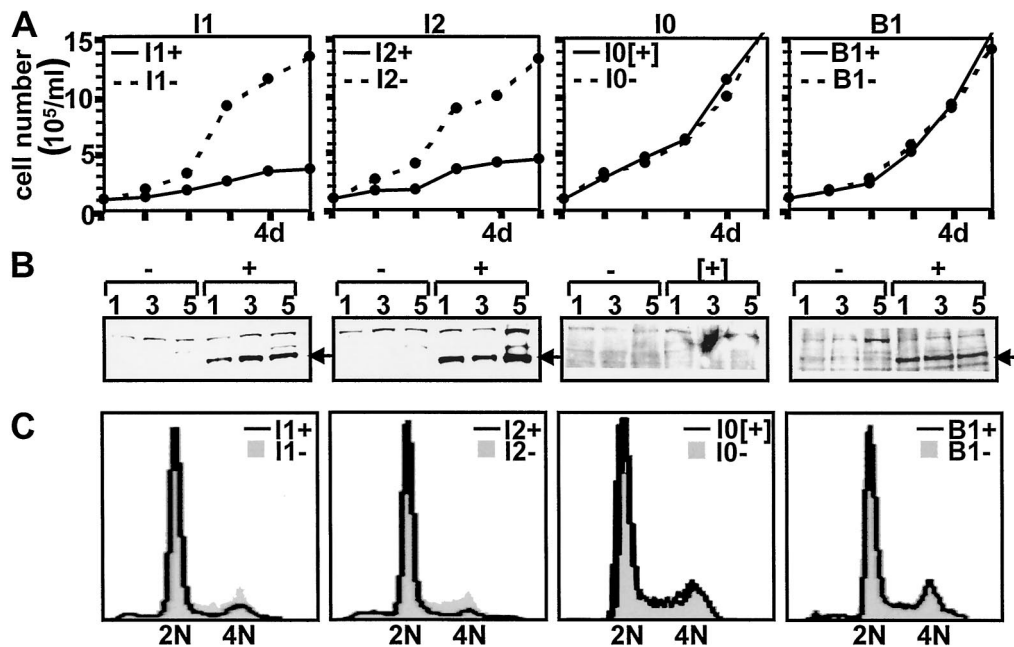


FIG. 2. Conditional FE3A overexpression causes growth arrest of IB4 LCL clones but does not affect Burkitt lymphoma growth. (A) Cell numbers of I1, I2, I0 and B1 cultures grown for 5 days under conditions of FE3A induction (+ or [+]; solid lines) or repression (-; dashed lines). [+], culture of the control IB4 clone I0, which does not conditionally express FE3A, under induction conditions; d, day. (B) Western blot with monoclonal antibodies M2 and M5, demonstrating FE3A expression (indicated by arrows) in I1, I2, and B1 cells under induced conditions (+) but not under repressed conditions (-) or in I0 cells under either condition at days 1, 3, and 5. (C) Cell cycle profiles of day 5 FE3A-induced cultures (I1+, I2+, I0[+], B1+) (open histograms with black borders) compared with those of FE3A-repressed cultures (I1-, I2-, I0-, B1-) growing at similar densities (shaded histograms without borders) as determined by propidium iodide staining and flow cytometry.

expression was induced (data not shown), which is characteristic of quiescent cells (26).

**Expression of a conditional *c-myc* restores S-phase entry.** To further explore the role of *c-myc* down-regulation in mediating cell cycle arrest in E3A-overexpressing IB4 cells, we attempted to restore growth with conditional expression of c-Myc fused to a mutant form of the estrogen receptor (MycERTM) that is activated by 4-HT and not by estrogen or phenol red (51). I2 cells stably transfected with mycER under control of the simian virus 40 promoter expressed substantially increased MycERTM levels within 2 days following addition of 4-HT to the culture; FE3A expression did not down-regulate mycER (Fig. 5A). After 4 days of FE3A induction and 2 days of MycERTM activation, ~30% more I2 cells were in S and G<sub>2</sub>/M than after FE3A induction without MycERTM activation (Fig. 5B). This indicates that MycERTM activation can restore cells to active cycle despite FE3A expression. However, cell densities did not substantially increase in the I2 cultures in which FE3A was induced at 2 or 4 days after MycERTM activation (Fig. 5D). Instead, the number of hypodiploid cells increased so that by day 4, 15% of the I2 cells with FE3A expression and MycERTM activation were hypodiploid and apoptotic, whereas I2 cells with FE3A expression and no MycERTM activation were only 5% hypodiploid (Fig. 5C). Interestingly, apoptosis was not an inevitable consequence of MycERTM activation; MycERTM activation in I2 cells in which FE3A was repressed resulted in fewer G<sub>0</sub>/G<sub>1</sub> cells and more-rapid increases in total cell densities than were seen in I2 cells that had FE3A repressed and no MycERTM activation (Fig. 5B and D). These

data indicate that the apoptosis-inducing effects of forced *c-myc* expression in I2 cells are dependent upon FE3A expression.

**E3A displaces E2 from RBP protein complexes.** Since E3A and E2 both associate with RBP and E2 up-regulates *c-myc* expression in LCLs, we investigated whether E3A overexpression in I2 cells affects E2 association with RBP. Under conditions of FE3A repression, immune precipitation of approximately 20% of E2 resulted in a similar level of RBP immune precipitation (Fig. 6). These data indicate that RBP is highly associated with E2 in I2 cells. Also, similar fractions of E2 and EBNA3C co-immune precipitated with RBP. In contrast, FE3A induction resulted in ~25% of the total overexpressed E3A in the RBP immune precipitate and little or no E2 (Fig. 6 and data not shown). Further, at least 50% of the RBP co-immune precipitated with FE3A, whereas RBP was not detected in the E2 immune precipitate. These data indicate that FE3A induction in I2 cells substantially disrupts E2 association with RBP. Interestingly, FE3A induction had less effect on EBNA3C association with RBP (Fig. 6), indicating that EBNA3C association with RBP is more stable than E2 association with RBP. These data indicate that three- to fivefold E3A overexpression in IB4 LCLs results in dissociation of E2 from RBP and continued association of EBNA3C with RBP.

**Genetic analyses of the E3A domain necessary for growth arrest.** To further evaluate the role of E3A association with RBP and E2 displacement in *c-myc* down-regulation and LCL growth arrest, we investigated whether E3A amino acids 1 to 277, which bind RBP in vitro (10, 82), are sufficient for down-

TABLE 1. Cell cycle distribution of FE3A-induced or -repressed I1, I2, I0, and B1 cells

Cells	FLAG-E3A <sup>a</sup>	% of cells <sup>b</sup> :			
		Apoptotic	In G <sub>0</sub> /G <sub>1</sub>	In S	In G <sub>2</sub> /M
I1	+	3	80	5	11
	-	1	59	19	20
I2	+	3	84	7	7
	-	2	64	20	14
I0	[+]	1	59	20	18
	-	1	60	21	17
B1	+	1	64	15	17
	-	1	66	14	18

<sup>a</sup> +, induced; -, repressed; [+], culture of the control IB4 clone I0, which does not conditionally express FE3A, under induction conditions.  
<sup>b</sup> Values are percentages of total cells from Fig. 2C with hypodiploid (apoptotic), 2N (G<sub>0</sub>/G<sub>1</sub>), S-phase, or 4N (G<sub>2</sub>/M) content.

regulation of E2 transcriptional activation, for stable association with RBP, for E2 dissociation from RBP, for *c-myc* down-regulation, and for LCL growth arrest. E3A deletion mutants and a triple alanine substitution mutant (T199A L200A C202A) that defines an RBP interaction domain within amino acids 125 to 222 in mammalian two-hybrid analysis (13) were tested in these assays. Wild-type FE3A inhibited E2 activation of luciferase transcription ~70% (Fig. 7B). Deletion of amino

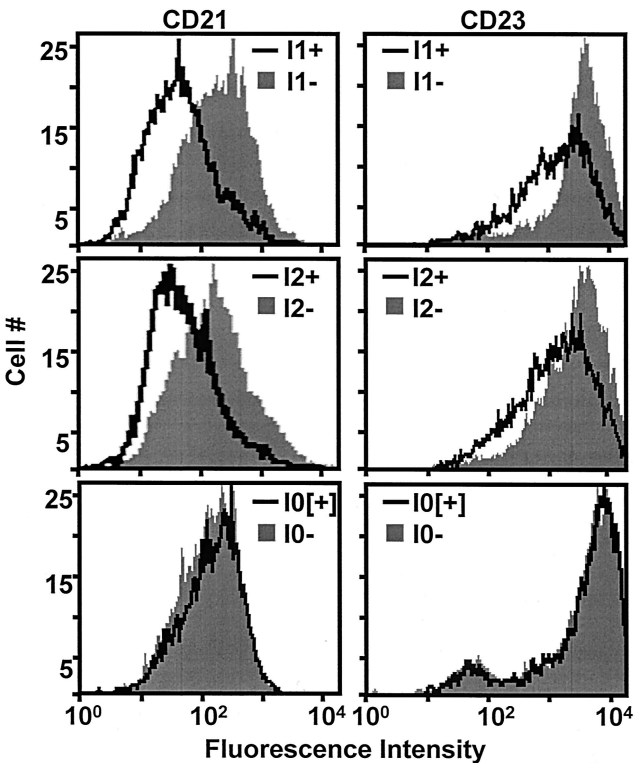


FIG. 3. Conditional FE3A overexpression reduces CD21 and CD23 cell surface expression in IB4 LCL clones. I1, I2, and I0 clones grown under conditions of FE3A induction (+ or [+]); open profiles with heavy outlines) or repression (-; shaded profiles) for 5 days were incubated with anti-CD21 or anti-CD23 monoclonal antibodies conjugated to phycoerythrin and analyzed by flow cytometry. [+], culture of the control IB4 clone I0, which does not conditionally express FE3A, under induction conditions.

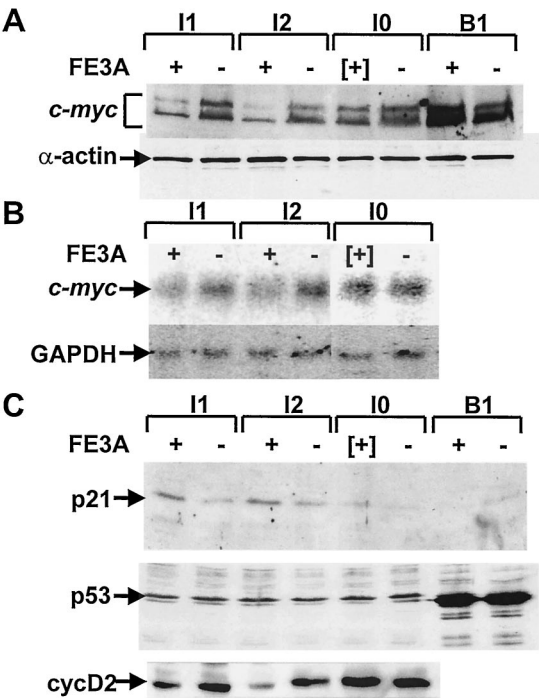


FIG. 4. FE3A overexpression in IB4 cells decreases *c-myc* and cyclin D2 expression and increases p21 expression but does not affect p53 expression. I1, I2, I0, and B1 cells were cultured under conditions for FE3A induction (+ or [+]) or repression (-). (A and B) *c-myc* expression at day 4 was analyzed by comparative Western (A) and Northern (B) blotting.  $\alpha$ -Actin or *gapdh* levels were analyzed as protein or RNA sample loading controls, respectively. (C) Comparative Western blots at day 4 with polyclonal antisera to p21, p53, or cyclin D2 (*cycD2*). [+], culture of the control IB4 clone I0, which does not conditionally express FE3A, under induction conditions.

acids 101 to 365, which include the RBP interaction domain and the nuclear localization sequence (46), resulted in cytoplasmic localization and no inhibition of E2 activation (Fig. 7B and data not shown). Surprisingly, FE3A 1-277 and FE3A 1-302, which include the RBP binding domain, were unable to inhibit E2 activation, whereas FE3A with amino acids 1 to 124 deleted, FE3A 1-386, or more C-terminally extended FE3A's were nearly wild type in effect (Fig. 7B). FE3A with amino acids 1 to 124 deleted, FE3A 1-277, FE3A 1-302, and FE3A 1-386 were similar to each other and somewhat lower than the wild type in protein levels (Fig. 7C). Thus, E3A amino acids 1 to 124 are not necessary for inhibiting E2 effects, and amino acids 1 to 277 or 1 to 302 are not sufficient for inhibiting E2 effects. Further, FE3A with the AAA substitution that substantially reduces RBP interaction did not inhibit E2 activation. These data indicate that E3A interaction with RBP is essential for E2 inhibition and that amino acids C-terminal to the core RBP binding domain are required for inhibition. Deletion of the E3A C terminus, which includes a transcriptional activation domain (13), did not affect E3A activity. Also, the E3A repression domain (5), which is C-terminal to 386, was not required for E2 inhibition.

IB4 clones were then established with conditional expression of FE3A 1-277, FE3A 1-523, FE3A with amino acids 101 to 365 deleted, or FE3A with the AAA substitution. Wild-type

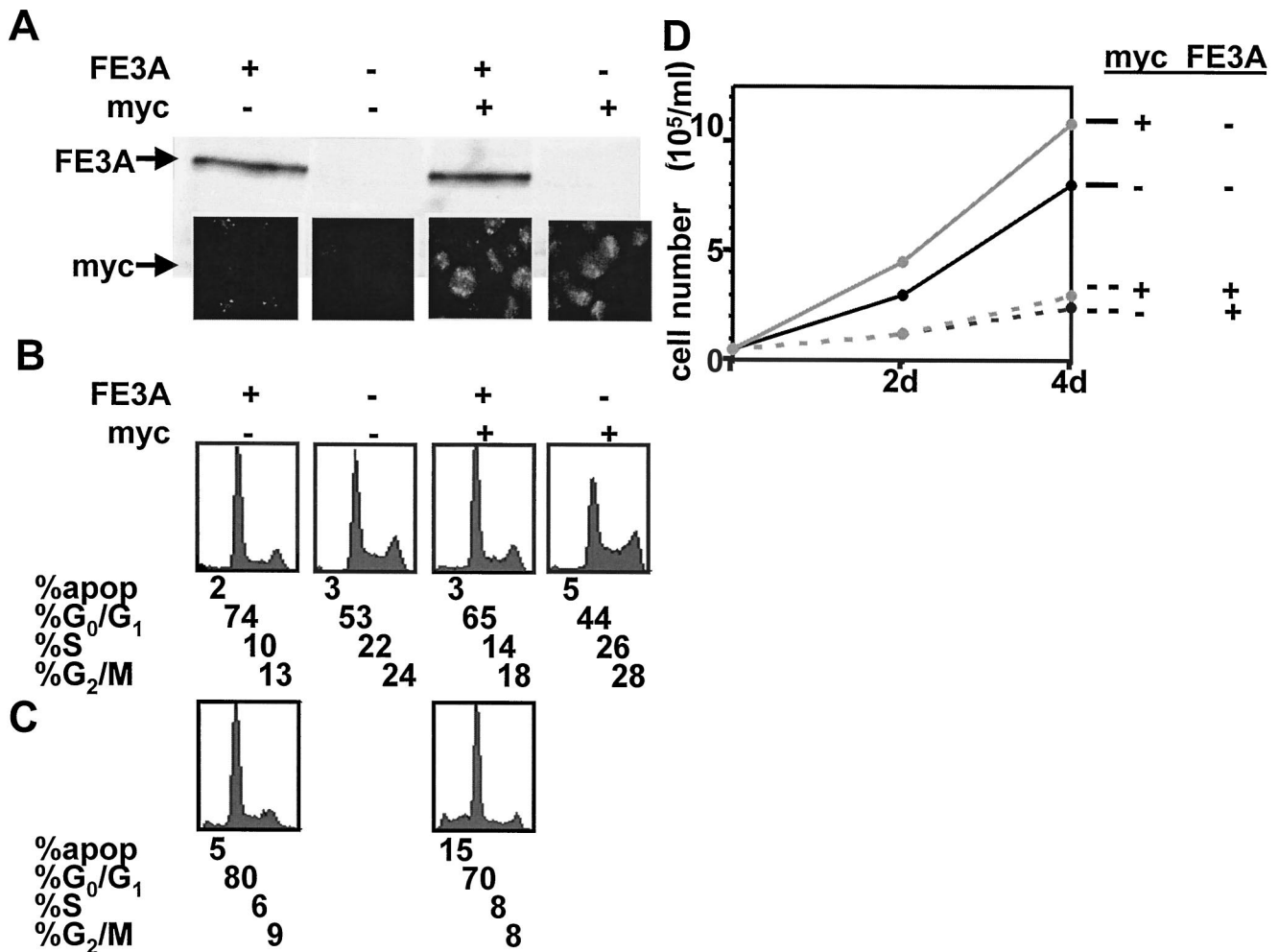


FIG. 5. Forced *c-myc* expression partially relieves FE3A-mediated growth arrest but increases apoptosis. Day 2 I2b cultures in which FE3A was induced (FE3A+) or repressed (FE3A-) were grown under conditions for either MycERTM activation (myc+) or MycERTM inactivation (myc-) for an additional 4 days. (A) FE3A expression was analyzed by Western blotting, and nuclear translocation of MycERTM was assessed by confocal microscopy 2 days after MycERTM activation. (B and C) I2b cells were analyzed by flow cytometry at day 2 (B) and day 4 (C) following MycERTM activation. Cell cycle profiles were examined to determine the percentage of total cells undergoing apoptosis (apop), in G<sub>0</sub>/G<sub>1</sub> phase, in S phase, or in G<sub>2</sub>/M phase. (D) Numbers of cells (10<sup>5</sup>/ml) of FE3A- and myc- (solid black line), FE3A+ and myc- (dashed black line), FE3A- and myc+ (solid gray line), or FE3A+ and myc+ (dashed gray line) cultures at 0, 2, and 4 days (d) following MycERTM activation.

and mutant FE3A expression was not detectable in cultures with TET and was induced within 2 days of TET withdrawal (data not shown). While, in general, the FE3A mutants were conditionally expressed at slightly lower levels than wild-type FE3A (Fig. 8B and data not shown), all were expressed at similar levels, and expression levels were adequate for a wild-type effect in the E2 inhibition assay (Fig. 7). Two clones of each FE3A mutant were selected for comparison with the wild type. Expression of FE3A with amino acids 101 to 365 deleted, FE3A 1-277, or FE3A with AAA substituted had no effect on cell growth or cell cycle distribution, whereas FE3A 1-523 was similar to the wild type in inhibiting cell growth and in causing increased numbers of G<sub>0</sub>/G<sub>1</sub> cells and decreased numbers of S and G<sub>2</sub>/M cells (Fig. 8A and 9A). I5 and I6 cultures in which FE3A 1-523 expression was induced routinely had approximately 2 and 4% more cells in the sub-2N population, respectively, than uninduced cultures, which was not substantially different from the 2% increases noted in IB4 clones in which

wild-type FE3A expression was induced and was much less than the 10% increase observed in cells with coexpression of conditional *c-myc* and FE3A (Table 1; Fig. 5C and 9A; data not shown).

Expression of FE3A with amino acids 101 to 365 deleted, FE3A 1-277, or FE3A with AAA substituted had no consistent effect on *c-myc* expression, whereas FE3A 1-523 was similar to the wild type in inhibiting *c-myc* expression (Fig. 9B). In other experiments, five clones of cells with varying levels of conditional overexpression of FE3A with the AAA substitution were compared to wild-type FE3A in FE3A expression levels, cell growth, and *c-myc* expression. Despite full wild-type induced expression levels in three clones, FE3A AAA did not affect *c-myc* expression or cell growth (data not shown). Thus, *c-myc* repression correlated with inhibition of E2 transcriptional activation, cell growth arrest, and G<sub>0</sub>/G<sub>1</sub> cell cycle arrest.

FLAG antibody was equally efficient in immune precipitation of induced wild-type FE3A, FE3A with amino acids 101 to



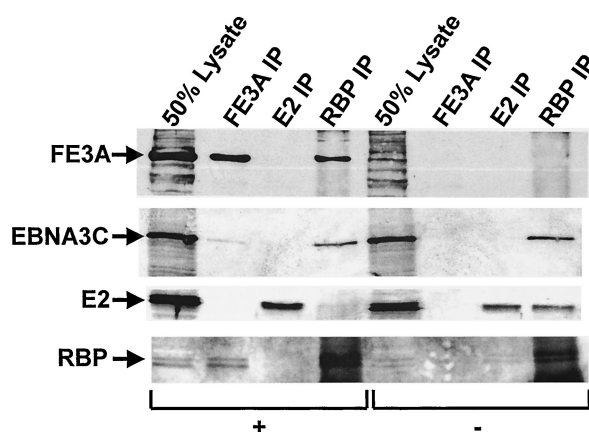


FIG. 6. Conditionally overexpressed FE3A is highly associated with RBP-J $\kappa$  (RBP) and inhibits the association of E2, but not EBNA3C, with RBP in IB4 cells. Protein complexes from I2 cells grown for 4 days under conditions of FE3A induction (+) or repression (–) were immunoprecipitated with antibodies to FLAG (FE3A IP), E2 (E2 IP), or RBP (RBP IP) and were Western blotted with FLAG-, E2-, EBNA3C-, or RBP-specific antibodies.

365 deleted, FE3A 1-277, FE3A 1-523, or FE3A with the AAA substitution from LCL extracts (Fig. 10). Wild-type FE3A and FE3A 1-523 immune precipitates had high levels of co-immune-precipitated RBP, whereas FLAG immune precipitates from cells expressing FE3A 1-277 or FE3A with amino acids 101 to 365 deleted, or from cells without FE3A overexpression, had no RBP (Fig. 10). Interestingly, FE3A with the AAA substitution had about 30% of the RBP that was present in wild-type FE3A or FE3A 1-523 immune precipitates (Fig. 10). Similarly, RBP immune precipitates had high levels of wild-type FE3A and FE3A 1-523 but barely detectable E2, whereas FE3A with amino acids 101 to 365 deleted and FE3A 1-277 did not co-immune precipitate with RBP. E2 was as abundant in the RBP immune precipitates from lysates of LCLs in which expression of FE3A with amino acids 101 to 365 deleted or FE3A 1-277 was induced as from nonexpressing cells. Despite the more efficient immune precipitation of RBP from LCLs in which FE3A with the AAA substitution was induced in the experiment for which results are shown in Fig. 10, FE3A AAA was less abundant than FE3A in the RBP immune precipitate and E2 was substantially more abundant. In experiments with other LCL clones that were induced to express FE3A with the AAA substitution, immune precipitation of FE3A AAA resulted in ~50% of the RBP that co-immune precipitated with similar amounts of wild-type FE3A, and immune precipitation of RBP resulted in ~50% co-immune precipitation of FE3A AAA relative to wild-type FE3A (data not shown). E2 co-immune precipitation with RBP from lysates of cells in which FE3A AAA expression was induced was also ~50% that from lysates of cells in which FE3A expression was repressed (Fig. 10 and data not shown). Thus, 50% of wild-type stable E2 association with RBP appears adequate to sustain wild-type LCL *c-myc* expression and cell proliferation.

## DISCUSSION

These experiments were initially undertaken to investigate the importance in LCL outgrowth of E3A association with the

DNA sequence-specific cellular transcription factor RBP. In LCLs, E2, E3A, and EBNA3C, which are essential for LCL outgrowth, and EBNA3B, which is nonessential, associate stably with most of the cellular RBP, while EBNA1P, which is also critical for LCL outgrowth, augments E2 transcriptional up-regulation (28, 39, 54, 61, 66, 67). While E2 and EBNA1P have strong transcriptional up-regulatory effects through E2 interaction with RBP, E3A, EBNA3B, and EBNA3C appear to modulate E2 activity at promoters through their activation or repression domains or to modulate E2 activity overall through their stable association with a substantial fraction of the total cellular RBP (5, 10, 13, 49, 83). In initial B-lymphocyte infection, E2 and EBNA1P up-regulate the EBNA Cp and probably the Wp promoter, resulting in increased transcription of E2 and EBNA1P and the turning on of E3A, EBNA3B, and EBNA3C (43). E3A, EBNA3B, and EBNA3C associate with RBP and have an imputed role in competing with E2 for access to RBP. Thus, RBP is a mediator of promoter-specific transcriptional effects of five of the six EBV latent infection nuclear proteins and a keystone for EBV nuclear protein transcriptional effects relevant to B-lymphocyte growth and survival.

E3A, EBNA3B, and EBNA3C competition with E2 for RBP availability is an attractive model for limiting E2 enhancement of Cp or other EBNA2-dependent cell or viral promoters. However, the precise role of RBP in the regulation of E2-responsive promoters has not been previously investigated in the context of latency III EBV infection in an LCL. E2 effects are more complicated. E2 has critically important interactions with other transcription factors in the regulation of the LMP1 and EBNA Cp promoters, and similar interactions may characterize E2 regulation of other viral and cellular promoters (21, 38). Although RBP is important for E2 up-regulation of the LMP1 promoter, the LMP1 promoter is more dependent on the B-cell and macrophage ets family DNA sequence-specific transcription factor PU.1 than on RBP. Moreover, E3A, EBNA3B, and EBNA3C can inhibit or potentiate E2 transactivation in various transient transfection assays (5, 13, 47, 49, 72, 82, 83). Thus, assessment of E3A effects in LCLs is necessary for understanding the role of E3A in the regulation of E2 transcriptional activation in LCLs.

We now find that conditional, three- to fivefold E3A overexpression in IB4 LCLs down-regulates *c-myc* and arrests IB4 LCLs in G<sub>0</sub>/G<sub>1</sub> without affecting endogenous EBNA or LMP1 expression. IB4 LCLs were used because EBNA expression was unlikely to be affected; the EBV genome in IB4 is integrated into chromosomal DNA and has the Cp enhancer deleted (31, 37, 76). Previous experiments also raise questions about the role of the E2-dependent Cp enhancer, since EBV recombinants with a mutated Cp RBP binding site are still competent for transformation, and EBNA expression in the resulting LCLs is similar to that of the wild type (16). The integrated IB4 EBV genome EBNA promoter is also insensitive to expression of a dominant-negative EBNA1, which down-modulates expression from an introduced oriP episome in IB4 LCLs (41). The stability of LMP1 expression in IB4 LCLs observed here with conditional E3A expression is fortuitous and is likely due to the proximity of the alternate LMP1 promoter to cellular DNA at the EBV genome integration site in the IB4 LCL (23, 55, 62). Also, E3A overexpression may potentiate E2 effects on the LMP1 promoter (49).

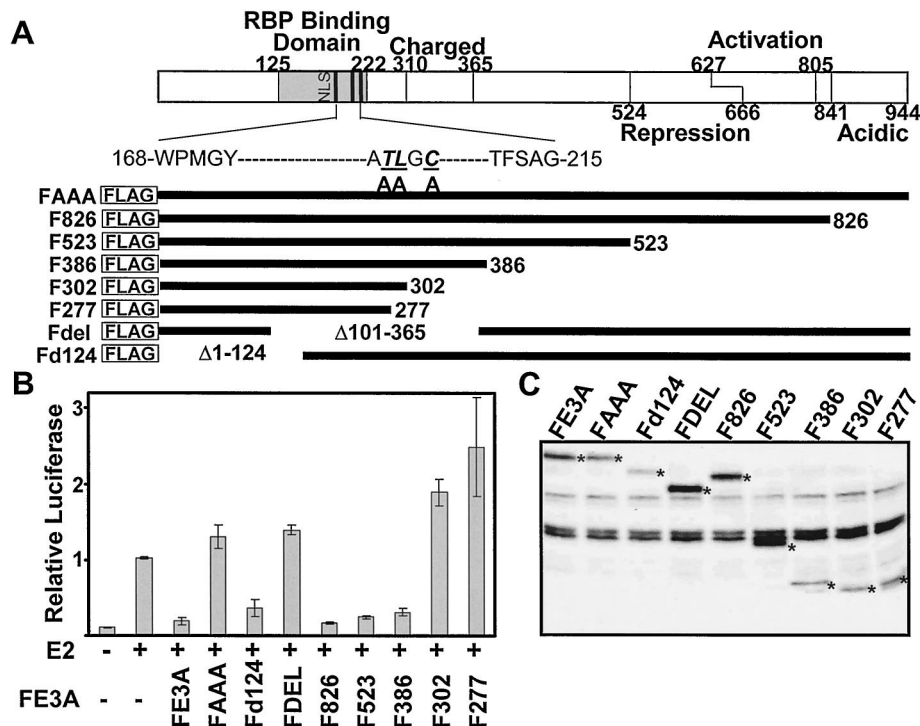


FIG. 7. The E3A RBP binding domain is necessary, but not sufficient, for inhibition of E2 transcriptional activation of a multimerized RBP binding site. (A) Features of E3A including the RBP binding domain (amino acids 125 to 222), the nuclear localization signal (NLS), residues (T199, L200, and C202) that are critical for RBP binding, the charged domain (amino acids 310 to 365), domains that repress (amino acids 524 to 666) or activate (amino acids 627 to 805) transcription when targeted to promoters, and the acidic C terminus. FE3A deletion and substitution (T199A L200A C202A) mutants are shown below. (B) BJAB cells were transfected with the pLucCp reporter construct containing 8 copies of the RBP binding site along with E2 (E2+) alone or with the indicated FE3A construct, and activation of transcription by E2 was evaluated by average relative luciferase activity from three independent experiments. (C) Expression of wild-type FE3A and FE3A mutants by Western blotting with monoclonal antibodies M2 and M5. Asterisks indicate the position of each immunoreactive band. Similar results were obtained from several experiments, and results of a representative experiment are shown.

The observations that conditional threefold overexpression of E3A in IB4 LCLs caused E2 dissociation from RBP, down-regulation of CD21, CD23, and *c-myc*, and  $G_0/G_1$  growth arrest despite wild-type levels of E2, EBNA1P, and LMP1 support the hypothesis that E3A can limit E2 association with RBP in LCLs and thereby inhibit E2 transcriptional effects. Threefold overexpression of E3A in IB4 LCLs is not far above physiological levels, since total EBNA3 protein levels probably increase less than twofold above wild-type LCL levels, given the absence of EBNA3B in IB4 LCLs (2). While E3A overexpression caused a dramatic decrease in E2 association with RBP, EBNA3C association with RBP was only slightly affected. Thus, in LCLs, EBNA3C is at least as effective as E3A in association with RBP, whereas E2 is substantially weaker.

The biochemical effects of conditional E3A overexpression in IB4 and the dependence of the effects described here on wild-type E3A or E3A 1-523, but not E3A 1-277 or E3A with the AAA mutation, indicate that RBP is a key mediator of E2 up-regulation of CD21, CD23, and *c-myc* in LCLs. Previous data implicate E2 and EBNA1P in the up-regulation of CD21, CD23, and *c-myc* expression in the first 24 h after EBV infection of B lymphocytes or after E2ER reactivation in an E2ER-dependent LCL, even in the absence of new protein synthesis (1, 40). The CD21 and CD23 promoters also have functional RBP binding sites and respond to activated Notch, another

up-regulator of transcription through RBP, under various conditions, including E2ER inactivation in an LCL (25, 36, 52, 65, 80). However, Notch IC-mediated induction of *c-myc* expression and LCL proliferation has only recently been observed in a single long-term clone of LCLs with high-level Notch IC after E2ER withdrawal (25). We note the presence of putative CTGGGAA RBP binding sites (60) in the human *c-myc* 5' control region (-3085) and in the first exon (+257), and a consensus GTGGGAA RBP binding site in the first intron. Elements just upstream of this RBP consensus site in *c-myc* intron1 negatively regulate *c-myc* transcription (8, 9, 33, 79, 81). Additionally, an RBP binding site in the first intron of CD21 has a critical role in lineage-specific silencing, raising the possibility of a similar role for RBP binding in the *c-myc* intron (52).

The differential effects of conditional wild-type and AAA mutant E3A on *c-myc* expression in IB4 cells with unperturbed EBNA and LMP1 expression may permit identification of a class of E2-regulated cell genes that are particularly dependent on RBP for transcriptional activation, versus PU.1, AUF1, AML1, c-jun/ATF2, or other transcription factors (21, 38, 63). E2 with a WW-to-SS mutation in the critical RBP interaction domain has 50% of wild-type E2 effect on the LMP1 promoter but results in complete loss of transcriptional activation by E2 on the Cp promoter (77). Thus, the LMP1 promoter is substantially less dependent on E2 transactivation through RBP



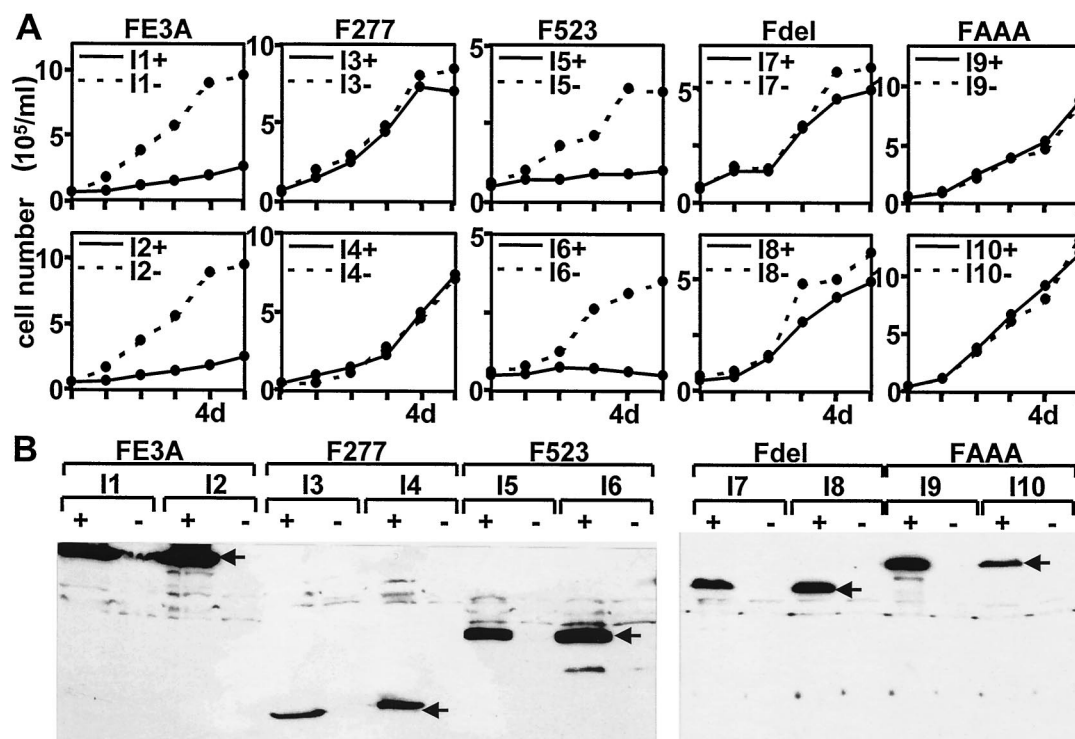


FIG. 8. The E3A RBP binding domain is necessary, but not sufficient, for IB4 growth arrest by conditional overexpression of FE3A. IB4 clones with conditional expression of FE3A (I1 and I2), FE3A 1-277 (F277; I3 and I4), FE3A 1-523 (F523; I5 and I6), FE3A  $\Delta$ 101-365 (Fdel; I7 and I8), and FE3A AAA (FAAA; I9 and I10) were analyzed for the number of viable cells over 5 days of induction (+; solid lines) or repression (-; dashed lines) (A) and for conditional expression at day 4 by Western blotting with FLAG epitope-specific antibodies M2 and M5 (B). FE3A, F277, F523, Fdel, and FAAA bands are indicated by arrows.

than the Cp promoter. Comparison in IB4 LCLs of the transcriptional effects of a putative dominant-negative E2 that is mutant in the acidic transactivation domain with the transcriptional effects of wild-type and AAA mutant E3A overexpression will provide an indication of whether RBP is equally critical for all transcriptional activating effects of E2. In contrast, RBP-dependent cell genes are expected to be relatively unaffected by overexpression of E2 that is doubly mutant in the acidic activating domain and the RBP interaction domain.

The prolonged viability of FE3A growth-arrested IB4 cells contrasts with the apoptosis observed with E2 inactivation in E2ER LCLs despite down-regulation of *c-myc* in both systems. Continued LMP1 expression and downstream NF- $\kappa$ B activation in the FE3A-overexpressing IB4 LCLs and down-regulation of LMP1 in E2ER LCLs likely account for at least part of this difference (25). However, MycERTM activation in LCL cells that were growth arrested as a consequence of FE3A overexpression partially restored S-phase entry and resulted in apoptotic cell death, whereas MycERTM activation in these cells in the absence of E3A overexpression did not cause apoptosis. Furthermore, forced *c-myc* expression in E2ER LCLs following E2ER withdrawal restored both viability and proliferation without LMP1 expression (58). These results suggest that the balance of pro- and antiapoptotic genes may be differentially affected by E2 and by E2 through RBP. An E2 RBP-dependent protein may be missing and required to prevent apoptosis with MycERTM activation in E3A-overexpress-

ing IB4 cells in the context of an E2-dependent and RBP-independent proapoptotic protein.

In supporting a role for RBP in E2-mediated *c-myc* up-regulation, our data also lend tangential support to the possibility that transcriptional activation of *c-myc* can be downstream of Notch1 activation, as was recently observed in a clone of E2ER-inactivated LCLs. Constitutively activated forms of Notch1 are associated with human T-cell leukemia, and retrovirus-mediated expression of activated Notch IC in mouse bone marrow can cause T-cell leukemia (3, 15, 17, 57). Notch IC certainly activates transcription through RBP, and RBP has a critical role in cell fate determinations (4, 6). In the absence of Notch or E2 activation, RBP is bound at promoter sites and silences gene expression by constitutive recruitment of the SKIP-SMRT-mSin3A corepressor complex and histone deacetylases (35, 42, 84, 85). Furthermore, activated Notch2 in B-lymphocyte chronic lymphocytic leukemia can activate CD23 and inhibit apoptosis (36). However, PU.1 or other transcription factor sites in the human *c-myc* promoter may specifically enable E2 to activate *c-myc* through RBP, and Notch IC may activate transcription through factors in addition to RBP (3). Indeed, activation of Notch1 can collaborate with *c-myc* overexpression in aggressive tumor induction in a mouse thymoma model (24, 34), suggesting that Notch1 affects pathways other than *c-myc* activation. Moreover, activating mutations of Notch1 are similar to *pim1* in cooperating with E2-

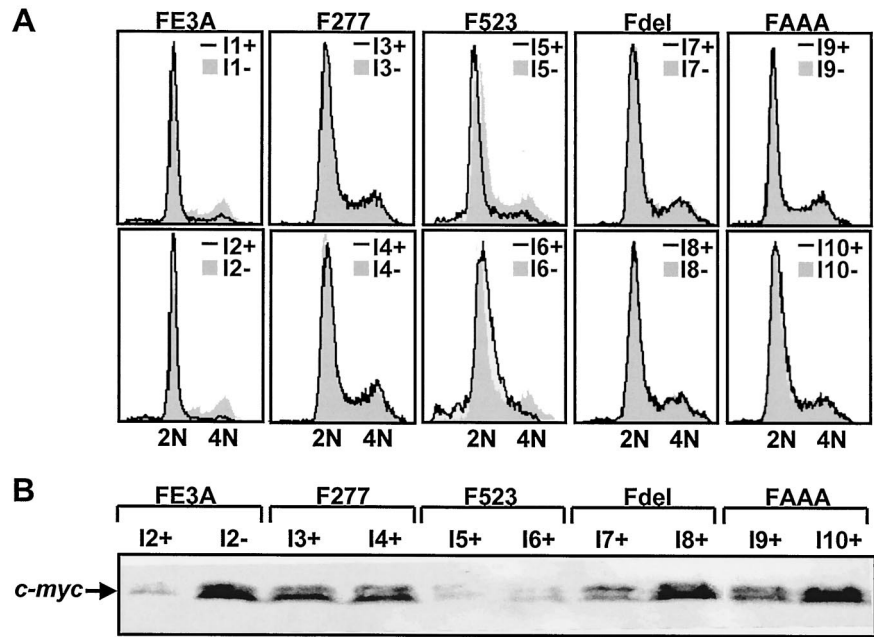


FIG. 9. G<sub>0</sub>/G<sub>1</sub> arrest of IB4 cells with conditional overexpression of FE3A or F523 correlates with loss of *c-myc* expression. (A) Comparison of cell cycle profiles of IB4 clones I1 to I10 cultured for 4 days under conditions for induction (+; open profiles with heavy outlines) or repression (-; shaded profiles) of FE3A or the indicated FE3A mutant by flow cytometry. (B) Western blot of *c-myc* expression in cells in which FE3A, F277, F523, Fdel, or FAAA was induced (+) compared to I2 cells in which FE3A was repressed (I2-) after 4 days in culture.

PBX1 in murine leukemogenesis (17, 18). The essential mechanisms of Notch1 and *pim1* complementation remain uncertain; *c-myc* enhancement could be an important effect. Pim1 phosphorylates p100, a regulator of c-Myb and of the E2 acidic

activation domain; phosphorylated p100 may enhance c-Myb activation of *c-myc* (14, 48, 69).

Our data add to the evidence that E3A charged amino acids 310 to 365 are important for stable E3A association with RBP in LCLs. In vitro, E3A amino acids 1 to 224 are sufficient for RBP binding (10, 82). In mammalian two-hybrid assays, E3A amino acids 125 to 222 are sufficient for RBP binding and E3A 125-222 with the AAA mutation lacks RBP binding activity (13). However, E3A amino acids 1 to 277 did not inhibit E2-mediated transactivation in our transient transfection studies, and conditionally overexpressed FE3A 1-277 in IB4 cells did not associate with RBP, did not affect E2 association with RBP, and had no effect on *c-myc* expression or cell growth. In contrast, E3A amino acids 1 to 386 or 1 to 523 were similar to wild-type E3A in transient assays of inhibition of E2 transactivation. Furthermore, conditional overexpression of E3A 1-523 in IB4 cells had full wild-type effects through RBP association, and E3A AAA only partially inhibited E2 association with RBP. These findings implicate the E3A RBP binding domain and charged amino acids 310 to 365 in stable E3A association with RBP and in competitive inhibition of E2 association with RBP. The E3A charged domain is conserved in EBNA3B and -3C, in EBV type II E3A, E3B, and E3C, and in EBNA3A homologues from herpesvirus papio and rhesus lymphocryptovirus, suggesting that this domain is important for stabilizing E3A association with RBP through intra- or inter-molecular interactions.

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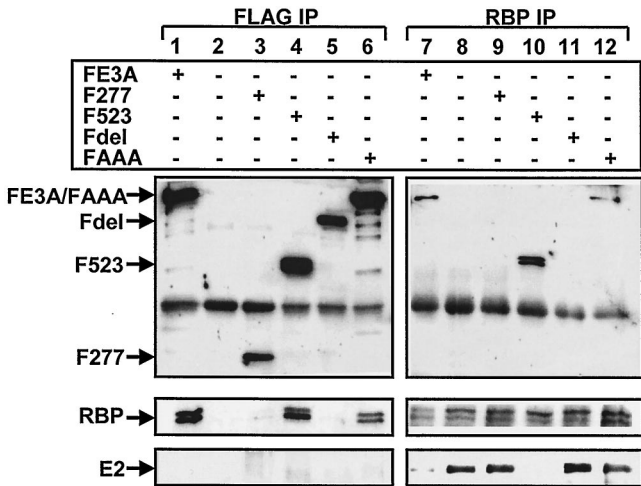


FIG. 10. Inhibition of E2 association with RBP in IB4 cells by conditional FE3A or F523 overexpression but not by FE3A mutants that fail to arrest IB4 cell growth. I2 cells grown under conditions for FE3A repression (lanes 2 and 8) or I2, I3, I5, I7, and I9 cells cultured for 4 days under conditions for induction of FE3A (lanes 1 and 7), F277 (lanes 3 and 9), F523 (lanes 4 and 10), Fdel (lanes 5 and 11), or FAAA (lanes 6 and 12), respectively, were harvested. Protein complexes were immunoprecipitated with monoclonal antibody M2 to the FLAG epitope (FLAG IP, lanes 1 to 6) or with a rabbit polyclonal antiserum to RBP (RBP IP, lanes 7 to 12) and were analyzed by immunoblotting with FLAG (top panel)-, RBP (middle panel)-, or E2 (bottom panel)-specific antibodies.

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